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ANTIBACTERIAL ACTIVITY AND FORCED DEGRADATION STUDY OF *CAESALPINIA SAPPAN* L. HEARTWOOD EXTRACT FOR INHIBITING PUS-FORMING BACTERIA

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Abstract: The aim of this research was to examine the antibacterial activity of *Caesalpinia sappan* heartwood extract against pus-forming bacteria. Furthermore, forced degradation of the extract was also investigated. *C. sappan* heartwood extract was prepared by maceration using 95% ethanol and decoction using boiling water. The antibacterial activity of extract was determined against *Staphylococcus aureus* TISTR 1466, *Staphylococcus epidermidis* TISTR 518 and *Propionibacterium acne* DMST 14916. The result found that the 95% ethanol extract of *C. sappan* inhibited bacterial growth with an inhibition zone of 20.40±1.64, 33.37±0.51, and 34.48±2.01 mm, respectively. The result found that the boiling water extract inhibited bacterial growth with an inhibition zone of 21.40±0.17, 30.40±0.00, and 57.90±1.20 mm, respectively. The forced degradation test found that 95% ethanol extract was decreased the antibacterial activity by neutral hydrolysis, acid hydrolysis, and thermal hydrolysis. In the case of boiling water extract, decreasing of antibacterial activity found in neutral hydrolysis, acid hydrolysis, and alkaline hydrolysis. In summary, *C. sappan* extract showed potent antibacterial activity. However, some condition caused decreasing of antibacterial activity should be avoided.

Keywords: *Caesalpinia sappan*, antibacterial activity, forced degradation

บทคัดย่อ: งานวิจัยนี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ต้านแบคทีเรียที่ก่อให้เกิดสิวหนองของสารสกัดจากแก่นฝางและการทดสอบด้วยการเร่งการเสื่อมสลาย สารสกัดจากแก่นฝางเตรียมได้จากการหมักด้วยเอทานอลความเข้มข้นร้อยละ 95 และการต้มในน้ำเดือด การศึกษาฤทธิ์ต้านแบคทีเรียของสารสกัดจะใช้แบคทีเรีย 3 ชนิด ได้แก่ *Staphylococcus aureus* TISTR 1466, *Staphylococcus epidermidis* TISTR 518 และ *Propionibacteium acne* DMST 14916 ผลการทดสอบพบว่า สารสกัดฝางในเอทานอลความเข้มข้นร้อยละ 95 สามารถยับยั้งแบคทีเรียโดยมีค่าการยับยั้ง 20.40±1.64, 33.37±0.51 และ 34.48±2.01 มิลลิเมตร ตามลำดับ ในขณะที่สารสกัดฝางด้วยน้ำร้อนสามารถยับยั้งแบคทีเรียโดยมีค่าการยับยั้ง 21.40±0.17, 30.40±0.00, และ 57.90±1.20 มิลลิเมตร ตามลำดับ การศึกษาการเร่งการเสื่อมสลายพบว่า สารสกัดจากเอทานอลความเข้มข้นร้อยละ 95 มีฤทธิ์ลดลงเมื่อผ่านการไฮโดรไลซิสในสภาวะกลาง สภาวะกรด และอุณหภูมิ ในกรณีของสารสกัดจากน้ำร้อน พบว่า มีฤทธิ์ลดลงเมื่อผ่านการไฮโดรไลซิสในสภาวะกลาง กรด และด่าง การศึกษานี้สามารถสรุปได้ว่า สารสกัดจากแก่นฝางมีฤทธิ์ยับยั้งเชื้อที่ดี แต่อย่างไรก็ตามควรหลีกเลี่ยงสภาวะที่ทำให้ฤทธิ์ต้านเชื้อลดลงดังที่กล่าวมาแล้วข้างต้น

คำสำคัญ: ฝาง ฤทธิ์ต้านแบคทีเรีย การเร่งการเสื่อมสลาย

INTRODUCTION

Caesalpinia sappan L. is a medicinal plant found in both the Ayurveda and Chinese tradition medicines that have been used since ancient time (Harjit *et al*., 2016). *C. sappan* is a plant of Leguminosae family, called Brazil or Sappan wood. The heartwood of this plant is commonly used for extraction of red dye. The heartwood of *C. sappan* has long been used in Thai folk medicine to treat many diseases including tuberculosis, diarrhea, dysentery, skin infections, and anemia (Sireeratawong *et al*., 2010). The chemical composition of Sappan wood usually found phenolic components such as xanthone, coumarin, chalcones, flavones, isoflavonoids and brazilin. Brazilin is the major naturally occur compound in the *C. sappan* heartwood and is used as a red dye for staining in histological application (Bae *et al*., 2005). Brazilin has many biological activities including antibacterial, anti-inflammatory, anti-photoaging, hypoglycemic, vasorelaxant, anti-allergic, anti-acne, antioxidant, and anti-nuclease activity (Nilesh *et al*., 2015).

In Thailand, it is mostly used as a colorant in beverage, food, costume, and cosmetic (Wetwitayaklung *et al*., 2005). A decoction of its heartwood is used in Namya-utai solution which has anti-thirst and cardiotonic properties since ancient time. In the north of Thailand, especially in Chiang Mai, Nan and Lampang province, decoction of *C. sappan* heartwood is used as an anti-inflammation for the treatment of traumatic disease and arthritis (Saenjum *et al*., 2010). In Ayurveda, the heartwood is used for vitiated conditions of *pitta* such as rashes, burning sensation, peptic ulcers, excessive body heat, heartburn, and dyspepsia. It also used as a blood purifier and in the treatment of wound for relief pain and swelling caused by external injuries and improvement of complexion (Srilakshmi *et al*., 2010). Based on above evidence and the ethnobotanical importance of *C. sappan* heartwood, thus it was selected for this study. Therefore, the aim of this research was to study the forced degradation of *C. sappan* before formulate as products. Inhibitory effect of *C. sappan* extract to *Staphylococcus aureus* TISTR 1466, *Staphylococcus epidermidis* TISTR 518, and *Propionibacterium acne* DMST 14916 was also compared between before and after forced degradation.

MATERIALS AND METHODS

Plant sample and chemicals

C. sappan heartwood was purchased from Nakhon Pathom province in Thailand. Ethanol and hydrochloric acid were purchased from Mallinckrodt, USA. Sodium hydroxide was obtained from Merck, USA. Clindamycin was obtained from Himedia, India. Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Brain Heart Infusion (BHI) broth, Anaerocult ® A and agar were purchased from Merck, Germany.

Plant extraction

C. sappan plant was examined according to their botanical characteristics to identify the right species. *C. sappan* was pulverized using a grinder and sieved. Two extraction conditions were used; maceration for 7 days with 95% ethanol at ambient temperature and boiling water, three times for each condition. Filtrate obtained from extraction was filtered through a Whatman No. 1 filter paper. The ethanol was removed from filtrate using a rotary evaporator (Buchi, Switzerland). In the case of aqueous extract was lyophilized using freeze dryer (LGJ-18S, Develop-MC, China). The sample is kept in a sterile bottle and stored at -20^{o} C for future use. Percentage yield (%, w/w) of each extract was calculated as the ratio of the weight of the extract to the weight of crude sample powder.

Bacterial strains

The following microorganisms were tested: *Staphylococcus aureus* TISTR 1466, *Staphylococcus epidermidis* TISTR 518, and *Propionibacterium acne* DMST 14916. *S. aureus and S. epidermidis* were grown in a test tube containing sterile MHB at 37 °C for 24 hours and *P. acne* was grown in BHI in an anaerobic jar at 37 °C for 48-72 hours. Several colonies were collected with a sterile inoculating loop, transferred into sterile saline solution, and adjusted to the desired concentration using the McFarland standard.

Agar diffusion susceptibility testing

C. sappan extract was assessed against all bacteria using disc diffusion test (6 mm sterile paper discs). Clindamycin (10 µg/disc) and water were used as positive and negative control, respectively. Bacterial inoculum ($1.5x10^8$ CFU/mL) were swabbed over the entire surface of the MHA and BHI agar plate. A sterile paper disc was placed in the Petri dish. Then 30 µL of *C. sappan* (10 mg/ml) was released on the paper disc. The disc of *C. sappan* and clindamycin were placed on the plates and incubated at 37 °C for 24 hours and *P. acne* was grown in BHI in an anaerobic jar at 37 °C for 48-72 hours. A vernier caliper was used to measure the inhibition zone diameter. Each sample was tested in triplicate.

Forced degradation study

Neutral hydrolysis

A 150 µL deionized water was added to 50 mg *C. sappan* extract. The sample was incubated at 80 ^{o}C for 3 hours. After that, the sample was tested for antibacterial activity.

Acid hydrolysis

A 150 µL of 3 N hydrochloric acid was added to 50 mg *C. sappan* extract. The sample was incubated at 80 ^{o}C for 3 hours. After that, the sample was tested for antibacterial activity.

Alkaline hydrolysis

A 150 µL of 3 N sodium hydroxide was added to 50 mg *C. sappan* extract. The sample was incubated at 80 ^{o}C for 3 hours. After that, the sample was tested for antibacterial activity.

Thermal degradation

The 50 mg *C. sappan* extract was incubated at 80 ^{o}C for 3 hours. After that, the sample was tested for antibacterial activity.

Oxidative degradation

A 150 µL of 30%v/v hydrogen peroxide was added to 50 mg *C. sappan* extract. The sample was incubated at 80 ^{o}C for 3 hours. After that, the sample was tested for antibacterial activity.

RESULTS AND DISCUSSION

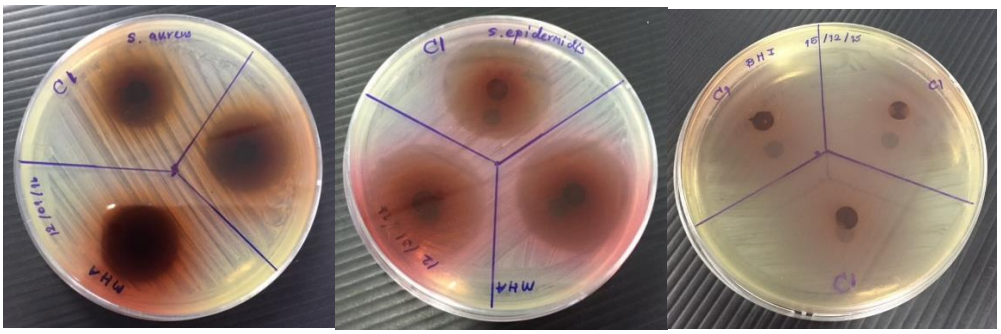
The dry powder of *C. sappan* was extracted with two different solvent systems using: 95% ethanol (by maceration) and boiling water (by decoction). The total yields were 9.44% and 6.71% when used 95% ethanol and water as solvents, respectively.

The research on antibacterial agents for the treatment pus-forming bacteria has become a very important endeavor. It has been reported that the crude extract of heartwood of *C. sappan* from screening 73 aqueous extracts displayed the best activity for *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 11490 (Xu and Lee, 2004). It has been reported the antimicrobial activities of *C. sappan* heartwood extracts were attributable to the presence mainly of polyphenols, which polyphenols are also known as antimicrobial compound (Guyot

et al., 1999). In the present study, *C. sappan* medicinal plant extracts were examined for antimicrobial activity against *S. aureus, S. epidermidis,* and *P. acne*. The results showed that extracts *C. sappan* (95% ethanol) and *C. sappan* (boiling water) could effectively inhibit the growth of *S. aureus, S. epidermidis, P. acne* and clindamycin showed a strong inhibitory effect in Table 1 and Figure 1-3, respectively. Ninety-five percent ethanol extract of *C. sappan* showed inhibited *S. aureus* as well as boiling water extract. However, clindamycin showed the best inhibition. In the case of *S. epidermidis,* boiling water extract had slightly lower inhibition zone compared to 95% ethanol extract. In addition, 95% ethanol extract showed similar inhibition zone compared to clindamycin. This result indicated that 95% ethanol extract of *C. sappan* had potent anti *S. epidermidis* activity. In the case of *P. acne,* 95% ethanol extract had lower inhibition zone compared to boiling water extract. Surprisingly, boiling water extract showed more potent anti *P. acne* compared to clindamycin.

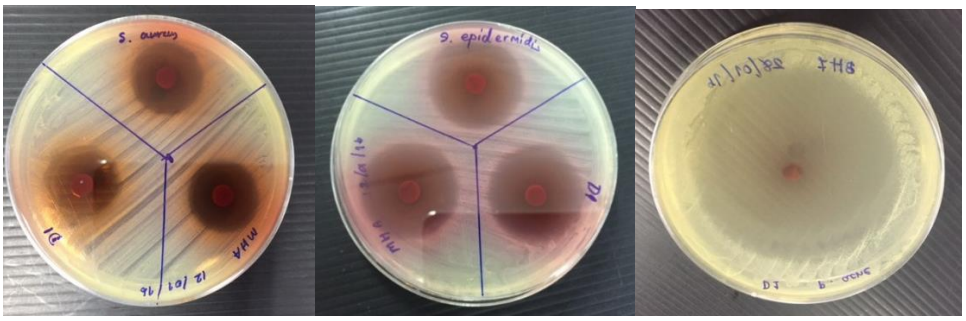
Table 1. The antibacterial activity of *C. sappan* extract against *S. aureus* TISTR 1466, *S. epidermidis* TISTR 518, and *P. acne* DMST 14916

Samples	Inhibition zone (mm)		
	S. aureus	*S. epidermidis*	*P. acne*
C. sappan extract (95% ethanol)	20.40±1.64	33.37±0.51	34.48±2.01
C. sappan extract (boiling water)	21.40±0.17	30.40±0.00	57.90±1.20
Clindamycin	30.00±0.00	33.33±2.87	54.95±0.05



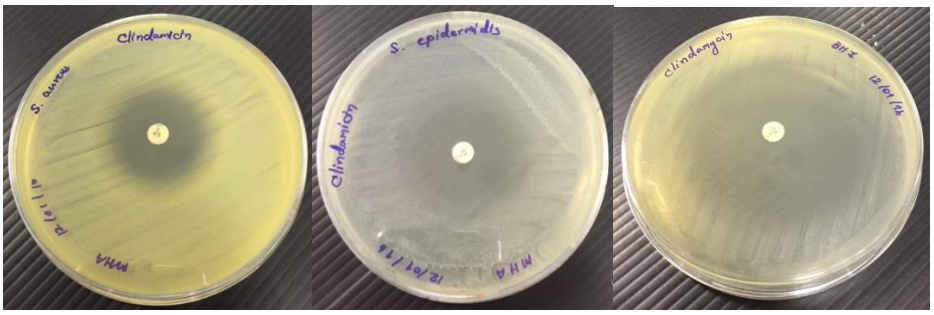
(a) **(b)** **(c)**

Figure 1. *C. sappan* extract (95 % ethanol) against (a) *S. aureus* TISTR 1466 (b) *S. epidermidis* TISTR 518 and (c) *P . acne* DMST 14916



(a) (b) (c)

Figure 2. *C. sappan* extract (boiling water) against (a) *S. aureus* TISTR 1466 (b) *S. epidermidis* TISTR 518 and (c) *P. acne* DMST 14916



(a) (b) (c)

Figure 3. Clindamycin against (a) *S. aureus* TISTR 1466 (b) *S. epidermidis* TISTR 518 and (c) *P. acne* DMST 14916

The forced degradation study in our work found that test the neutral and acid hydrolysis caused decreasing of the inhibitory effect of extract to all bacteria. Whereas, a test of alkaline hydrolysis showed that *C. sappan* extract (95% ethanol) could inhibit the growth of all bacteria higher than non-alkaline hydrolysis *C. sappan* extract (95% ethanol) but boiling water extract caused decreasing of the inhibitory effect of extract to all bacteria. In the case of thermal degradation, the result showed that inhibitory effect of extract of *C. sappan* (95% ethanol) was decreased for *S. aureus* and *S. epidermidis* but increased for *P. acne*. However, boiling water extract was not affected by the thermal degradation. In the case of oxidative degradation test, the result showed that the inhibitory effect of *C. sappan* extract (95% ethanol) was not affected but boiling water extract showed higher inhibition zone for *S. aureus* and *S. epidermidis.* Conversely, the inhibitory effect on *P. acne* was decreased. The result of forced degradation test is shown in Table 2. This result indicated that some conditions caused decreasing of antibacterial activity should be avoided to maintain the inhibitory effect of this plant extract throughout manufacturing process until patient use. Goossens reported that the active antibacterial principle in *C. sappan* that was purified and identified was brazilin. Brazilin is a colorless substance in its pure state and changes to red on exposure to air or mind alkaline oxidation (Goossens, 2000). Thus, the chemical test should be further investigated to clarified the correlation between a chemical compound and biological activity of the plant.

Table 2. Forced degradation study of *C. sappan* extract against *S. aureus* TISTR 1466, *S. epidermidis* TISTR 518, and *P. acne* DMST 14916.

Condition	Plant extract	Inhibition zone (mm)		
		S.aureus	*S.epidermidis*	*P.acne*
Neutral hydrolysis	*C. sappan* (95% ethanol)	18.93±1.00	31.17±1.11	27.77±0.75
	C. sappan (boiling water)	18.80±0.20	19.33±1.26	49.30±0.00
Acid hydrolysis	*C. sappan* (95% ethanol)	18.77±0.96	32.37±0.00	30.70±1.39
	C. sappan (boiling water)	18.20±0.35	25.23±0.58	22.93±0.99
Alkaline hydrolysis	*C. sappan* (95% ethanol)	24.20±0.35	33.77±1.15	58.23±0.80
	C. sappan (boiling water)	14.40±0.00	18.37±1.48	32.97±0.94
Thermal degradation	*C. sappan* (95% ethanol)	17.70±1.49	28.87±1.93	45.40±1.90
	C. sappan (boiling water)	20.93±0.70	32.47±1.93	54.97±0.61
Oxidative degradation	*C. sappan* (95% ethanol)	22.30±0.52	34.05±0.09	32.76±0.55
	C. sappan (boiling water)	27.73±1.16	32.13±0.11	24.63±0.66

CONCLUSION

This research found that *C. sappan* heartwood (95% ethanol and boiling water extract) had potent inhibition to three bacteria caused pus; *S. aureus*, *S epidermidis* and *P. acne.* The result of forced degradation study showed that anti-bacterial activity of 95% ethanol extract was decreased by neutral hydrolysis, acid hydrolysis, and thermal hydrolysis. For boiling water extract, decreasing of antibacterial activity found in neutral hydrolysis, acid hydrolysis, and alkaline hydrolysis.

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